PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
PAM-011-PCT		

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP 03/12058	30.10.2003	30.10.2002

International Patent Classification (IPC) or both national classification and IPC

C12Q1/68

Applicant

PAMGENE B.V. et al.

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 7 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the opinion
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
17.05.2004	09.02.2005

Name and mailing address of the international preliminary examining authority:	Authorized Officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Brenz Verca, S Telephone No. +49 89 2399-7702

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP 03/12058

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished t the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed' and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:

Description, Pages

1-8, 10-26	as originally filed
9	received on 15.11.2004 with letter of 09.11.2004

Sequence listings part of the description, Pages

1-2	as originally filed

Claims, Numbers

1-25	filed with telefax on 15.12.2004

Drawings, Sheets

1/1	as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: ; which is:

   ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).

   ☐ the language of publication of the international application (under Rule 48.3(b)).

   ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   ☒ contained in the international application in written form.

   ☒ filed together with the international application in computer readable form.

   ☐ furnished subsequently to this Authority in written form.

   ☐ furnished subsequently to this Authority in computer readable form.

   ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   ☐ the description, pages:

   ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)).

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to tl report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicabilit citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-25
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-25
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-25
	No:	Claims	

2. Citations and explanations

see separate sheet

Re Item V

Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

The application pertains to a two-step isothermal nucleic acid amplification system for the generation of multiple cRNA copies directly from an educt RNA without the intermediate synthesis of cDNA. An oligonucleotide primer containing in its 5' region a RNA polymerase promoter sequence and in its 3' region a sequence (random or arbitrary or predetermined) with a blocked 3' end and at least a chimeric linkage at its 3'-end, hybridizes to the educt RNA. An RNASE H enzyme digests the 3' free end of the educt RNA, a DNA polymerase activity elongates the educt RNA using the oligonucleotide as template, thereby incorporating the promoter sequence in form of DNA. Finally, a RNA polymerase synthesizes cRNA copies using the engineered educt RNA as template.

1. Reference is made to the following documents:

D1: WO 99/43850 A (AKZO NOBEL NV ;GEMEN B VAN (NL)) 2 September 1999 (1999-09-02)

D2: EP-A-0 721 988 (BIO MERIEUX) 17 July 1996 (1996-07-17)

D3: LOGEL J ET AL: "SYNTHESIS OF CRNA PROBES FROM PCR-GENERATED DNA" BIOTECHNIQUES, EATON PUBLISHING, NATICK, US, vol. 13, no. 4, 1992, pages 604-606,608, XP002947579 ISSN: 0736-6205

D4: DEIMAN BIRGIT ET AL: "Characteristics and applications of nucleic acid sequence-based amplification (NASBA)." MOLECULAR BIOTECHNOLOGY, vol. 20, no. 2, February 2002 (2002-02), pages 163-179, XP009007141 ISSN: 1073-6085

2. **Novelty (A33(2) PCT):**

2.1 **D1**discloses a similar method for the generation of multiple RNA copies employing an oligonucleotide comprising a T7 promoter sequence in the 5' region and a target hybridizing sequence in the 3' end which is a poly-dT stretch (**D1**: see passages cited in the International Search Report, in particular example 2). The oligonucleotide PH26 of example 2 in D1 is blocked at its 3' end by a biotin.

2.2 **D2** discloses a method for amplifying nucleic acids (see passages cited in the

International Search Report, in particular example 6, figure 1) using a DNA/RNA chimeric oligonucleotide which comprises a T7 promoter sequence (P1) on its 5' end, a target-hybridizing sequence (S1) on its blocked 3' end and a transcription initiation region (I1) in between. E.coli RNASE H (D2: p. 10, line 56; p. 8, lines 44-46), Klenow DNA Pol exo(-), T7 RNA polymerase are thereby used to produce cRNA copies, the predetermined sequence used for experiments is an RNA derived from the TEM gene coding for beta-lactamase (D2: p.10, lines 48-49) and is thus a "*gene-specific sequence*".

2.3 Present independent claims 1-3 differ from D1 and D2 by specifying the use of at least a chimeric linkage between nucleotides at the 3' end of the oligonucleotide and are therefore novel over the prior art. Furthermore, claim 2 differs from D2 by involving the use of a DNA oligonucleotide instead of a chimeric DNA/RNA oligonucleotide.

2.4 The kit of claim 23 comprises an oligonucleotide having as technical features either a random sequence or a predetermined sequence at the 3' end, a promoter sequence at the 5' end, an extension-blocking modification at its 3' end, at least a chimeric linkage between nucleotides at the 3' end, as well as instructions to carry out the method for generating multiple RNA copies according to the previous method claims. Prior art **D1** discloses an oligonucleotide (e.g. oligonucleotide PH26 of example 2) having the same features, except or the chimeric linkage between nucleotides at the 3' end, thus the kit of claims 23-25 is novel.

2.5 Prior art **D3** discloses several oligonucleotides for the generation of cRNA probes from PCR amplification products which comprise a phage polymerase promoter sequence on their 5' end linked to a gene-specific sequence on their 3' end (D3: table 1), however said oligonucleotides are not blocked at the 3'-end and do not have at least one chimeric linkage between nucleotides at the 3' end.

3. Inventive step (A33(3) PCT):

3.1 **D1** can be considered to be the closest prior art, disclosing a method for the non-specific amplification of poly A mRNA by generating cRNA copies without the generation of cDNA intermediates (see previous point 2.1).

3.2 With regard to independent claim 1, the difference from D1 is that the method of the invention employs an oligonucleotide comprising a random sequence as target hybridizing sequence at its 3' end, thus the method of the application does not amplify solely RNAs comprising polyA stretches. Furthermore, the oligonucleotide of claim 1 has at least one chimeric linkage between nucleotides at the 3' end, reducing its sensibility to nuclease degradation.

Thus, the problem to be solved can be formulated as the provision of a method for the efficient non-specific amplification of any kind of RNA without using cDNA intermediates, thereby reducing the sensibility to nucleases.

It is believed that the different possibilities to generate cDNA from RNA, e.g. the use of oligo dT primers, random hexamers or specific primers with their correspondingly linked advantages/disadvantages, belong to the general knowledge of a common practitioner in the art, who therefore knows that an oligo dT primer allows the generation of cDNA from RNAs with a poly A tail, while random hexamers allow the generation of cDNA also from other types of RNAs. However, the use of at least one chimeric linkage between nucleotides at the 3' end in order to stabilize the oligonucleotide was not suggested in the cited prior art.

3.3 With regard to independent claims 2 and 3, the difference from D1 is the use of an oligonucleotide comprising a chimeric linkage between nucleotides at the 3' end and having a ***predetermined*** target hybridizing sequence at its 3' end.

The problem to be solved over D1 was thus the provision of a method for the ***specific*** amplification of RNA without generating cDNA intermediates and the reduction of the sensibility to nucleases.

Again, the skilled person knew very well that specificity can be achieved by the choice of appropriate nucleotide sequences in oligonucleotides, therefore the mere fact of "providing specificity" over the method of D1 would not render said claims inventive. However, the use of a chimeric linkage was not suggested neither in D1, nor in D2.

3.4 Consequently, inventive step is acknowledged (A33(3) PCT).

3.5 The kit of claims 22-25 is linked to the method of claims 1-22 by the special technical feature of an oligonucleotide comprising the following features:

- a promoter sequence in its 5' region
- a target hybridizing sequence in its 3' end selected from a random sequence or a predetermined sequence
- an extension-blocking modification at its 3' terminal end
- at least one chimeric linkage between nucleotides at its 3' end;

and is specially adapted to the method of claims 1-21 by the inclusion of specific instructions. Inventive step in the light of the method of claims 1-21 is therefore acknowledged (see supra).

4. Industrial applicability (A33(4) PCT):

All claims appear to fulfill the requirement of industrial applicability of A33(4) PCT.

Claims (retyped)

1. A method for generating multiple RNA copies comprising the steps of:
   (a) providing a sample comprising target RNA, wherein said sample is simultaneously contacted with:
   - an oligonucleotide comprising at its 5' side a promoter sequence recognized by an RNA polymerase, wherein said oligonucleotide further comprises:
     - a target hybridising sequence, which is a random sequence,
     - a modified nucleotide at its 3' terminal end in such a way that extension therefrom is prohibited, wherein said modified nucleotide is chosen from the group comprising nucleotides comprising alkane-diol residues, cordycepins, amino-alkyls, and dideoxynucleotides,
     - at least one chimeric linkage between nucleotides at the 3' end; wherein said chimeric linkage may contain at least one phosphorothioate linkage between nucleotides, or a PNA, LNA or GripNA backbone, and;
   - an enzyme having DNA polymerase activity;
   - an enzyme having RNase H activity;
   - an enzyme having RNA polymerase activity; and,
   - sufficient amounts of nucleotides; and,

   (b) maintaining the resulting reaction mixture under the appropriate conditions for a sufficient amount of time for the enzymatic processes to take place.

2. A method for generating multiple RNA copies comprising the steps of:
   (a) providing a sample comprising target RNA, wherein said sample is simultaneously contacted with:
   - a DNA oligonucleotide comprising at its 5' side a promoter sequence recognized by an RNA polymerase, wherein said oligonucleotide further comprises:
     - a target hybridising sequence, which is a predetermined sequence,

- a modified nucleotide at its 3' terminal end in such a way that extension therefrom is prohibited, wherein said modified nucleotide is chosen from the group comprising nucleotides comprising alkane-diol residues, cordycepins, amino-alkyls, and dideoxynucleotides,
- at least one chimeric linkage between nucleotides at the 3' end; wherein said chimeric linkage may contain at least one phosphorothioate linkage between nucleotides, or a PNA, LNA or GripNA backbone, and;

- an enzyme having Klenow pol I exo (-) activity;
- an enzyme having RNase H activity;
- an enzyme having RNA polymerase activity; and,
- sufficient amounts of nucleotides; and,

(b) maintaining the resulting reaction mixture under the appropriate conditions for a sufficient amount of time for the enzymatic processes to take place.

3. A method for generating multiple RNA copies comprising the steps of:

(a) providing a sample comprising target RNA; wherein said sample is simultaneously contacted with:

- an oligonucleotide comprising at its 5' side a promoter sequence recognized by an RNA polymerase, wherein said oligonucleotide further comprises:
  - a target hybridising sequence, wherein said hybridising sequence is a predetermined sequence,
  - a modified nucleotide at its 3' terminal end in such a way that extension therefrom is prohibited, wherein said modified nucleotide is chosen from the group comprising nucleotides comprising alkane-diol residues, cordycepins, amino-alkyls, and dideoxynucleotides,
  - at least one chimeric linkage between nucleotides at the 3' end; wherein said chimeric linkage may contain at least one phosphorothioate linkage between nucleotides, or a PNA, LNA or GripNA backbone, and,
- an enzyme having DNA polymerase activity;
- an enzyme having RNase H activity;

- an enzyme having RNA polymerase activity; and,
- sufficient amounts of nucleotides; and,

(b) maintaining the resulting reaction mixture under the appropriate conditions for a sufficient amount of time for the enzymatic processes to take place.

4. The method according to any of claims 1 to 3, wherein said target RNA is of eukaryotic, prokaryotic or viral origin, or a mixture thereof.

5. The method according to any of claims 1 to 4, wherein said target RNA is chosen from the group comprising total RNA, mRNA, cRNA, rRNA, tmRNA, asRNA, hnRNA or tRNA, including any combination thereof.

6. The method according to any of claims 2 to 5, wherein said predetermined sequence is chosen from the group comprising gene-specific sequences, viral sequences, prokaryotic sequences, mutation-specific sequences, poly-T sequences, genomic sequences and rRNA.

7. The method according to any of claims 1 to 6, wherein at least one of the nucleotides, e.g. dNTPs and rNTPs, is provided with a label.

8. The method according to any of claims 1 to 7, wherein the generated RNA is used as input material for further amplification.

9. The method according to any of claims 1 to 8, wherein the generated RNA is contacted with:

- an RNA ligase,
- a double stranded nucleic acid complex comprising a double stranded DNA promoter sequence that can be recognized by an RNA polymerase, whereby one strand of said complex has a stretch of RNA attached to the 5' end of one of the DNA strands,

- an enzyme having RNA polymerase activity, and
- sufficient amounts of dNTPs and rNTPs;

wherein the resulting reaction mixture is maintained under the appropriate conditions for a sufficient amount of time for the enzymatic processes to take place.

10. The method according to any of claims 1 to 9, wherein the reaction mixture further comprises:
    - an RNA ligase; and,
    - a double stranded nucleic acid complex comprising a double stranded DNA promoter sequence that can be recognized by the RNA polymerase, whereby one strand of said complex has a stretch of RNA attached to the 5' end of one of the DNA strands.

11. The method according to any of claims 1 to 10, wherein the generated RNA copies are contacted with poly A polymerase.

12. The method according to any of the claims 1 to 11, wherein the starting material is simultaneously contacted with a poly A polymerase.

13. The method according to any of claims 1 to 12, wherein said promoter sequence is a T7 promoter sequence.

14. The method according to any of claims 1 to 13, wherein said RNA polymerase is a T7 RNA polymerase.

15. The method according to any of claims 1 and 3 to 14, wherein said enzyme having DNA polymerase activity is AMV-RT or MMLV-RT.

16. The method according to any of claims 1 to 15, wherein said enzyme having RNase H activity is *E. coli* RNase H .

17. The method according to any of claims 1 to 16, wherein said enzyme having RNase H activity is reverse transcriptase.

18. The method according to claim 17, wherein said enzyme having RNase H activity is AMV-RT or MMLV-RT.

19. A method for determining differences in gene expression in cell samples, comprising the steps of:
    - creating multiple RNA copies of one or more target RNA species according to the method of any of claims 1 to 18, whereby a first pattern of expression is formed from the sample;
    - comparing said first pattern of expression with a predetermined pattern of expression, whereby differences in gene expression are determined.

20. The method according to any of claims 1 to 19, wherein said multiple RNA copies are used to interrogate a probe array.

21. The method according to claim 20, wherein said probe array is an oligonucleotide array.

22. Kit for generating multiple RNA copies comprising:

an oligonucleotide comprising at its 5' side a promoter sequence recognized by an RNA polymerase, wherein said oligonucleotide further comprises a target hybridising sequence, which is a random sequence or a predetermined sequence, said predetermined sequence complexing predominantly with the intended target RNA to be amplified, a modification at its 3' terminal end in such a way that extension therefrom is prohibited, and at least one chimeric linkage between nucleotides at the 3' end, wherein said chimeric linkage may contain at least one phosphorothioate linkage between nucleotides, or PNA, LNA or GripNA backbone, and

- instructions to carry out the method according to any of the Claims 1 to 21 for generating multiple RNA copies.

23. The kit according to claim 22, further comprising:

- an RNA ligase,
- a double stranded nucleic acid complex comprising a double stranded DNA promoter sequence that can be recognized by an RNA polymerase, whereby one strand of said complex has a stretch of RNA attached to the 5' end of one of the DNA strands, and
- instructions to carry out further amplification.

24. The kit according to claim 22 or 23 , further comprising a probe array.

complementary sequences, but also on the hybridisation conditions. The hybridisation conditions, such as hybridisation time, temperature, wash buffers used, etc. can be altered to optimise the efficient and specific binding of the target sequences. Suitable hybridisation conditions for various nucleic acid pairs are well known to those skilled in the art and reviewed in e.g. Sambrook et al., 1989 (in "Molecular Cloning: a laboratory manual" 2nd edition; Cold Spring Harbor Laboratory Press, USA), which is herein specifically incorporated by reference. The terms "hybridise" and "hybridisation" refer to the formation of complexes between nucleotide sequences which are sufficiently complementary to form complexes via Watson-Crick base pairing. Where an oligonucleotide, i.e. the "target hybridising sequence", "hybridises" with target RNA (template) at the target sequence, such resulting complexes (or hybrids) are sufficiently stable to serve the priming function required by DNA polymerase, e.g. reverse transcriptase or Klenow pol I exo (-), to initiate DNA synthesis.

In an embodiment, the invention provides also a method for generating multiple RNA copies comprising the steps of:

(a) providing a sample comprising target RNA, wherein said sample is simultaneously contacted with:
- an DNA oligonucleotide, ~~preferentially a DNA oligonucleotide~~, comprising at its 5' side (possibly at its 5' end) a promoter sequence recognized by an RNA polymerase, wherein each oligonucleotide further comprises a target hybridising sequence, which is a predetermined sequence; and,
- an enzyme having Klenow pol I exo (-) activity;
- an enzyme having RNase H activity;
- an enzyme having RNA polymerase activity; and,
- sufficient amounts of dNTPs and rNTPs; and,

(b) maintaining the resulting reaction mixture under the appropriate conditions for a sufficient amount of time for the enzymatic processes to take place.

Upon hybridisation of the oligonucleotide to the target RNA, the target RNA is cut by an enzyme having RNase H activity, which generates a new 3' end of the target RNA. The newly generated 3' end of the RNA is extended by an enzyme having DNA polymerase activity, e.g. reverse transcriptase or Klenow pol I exo (-), on the oligonucleotide template to generate a double stranded promoter sequence. Hence, the enzyme having DNA polymerase activity